REMARKS/ARGUMENTS

This application was originally filed with claims 1-21. Claims 15-21 have been previously cancelled. In this response, claims 1, 2, 8, 11 and 13 have been amended. Claim 12 has been cancelled herein without prejudice. New claims 22-32 have been added. Specific support for these amendments is found in the as-filed specification. Specifically, the generation of directed genetic modification is provided in the specification at, for example, [0039], [0096]-[0097] and [0112] for example. The development of compensatory metabolic pathways is discussed in the specification at, for example, [0250], [0302], [0364], [0369]-[0373] and [0530] as well as being specifically the topic of Examples F.1 and F.1.2. The development of such compensatory metabolic pathways is further specifically illustrated in Figures 2 and 4. Thus, no new matter is added by way of these amendments.

Examiner Interview Summary

On Monday June 23, 2008, Inventor Philippe Soucaille and attorneys Colin Fairman and Franck Tetaz conducted a personal interview with Examiners Shahnan-Shah and Foley. Dr. Soucaille explained the invention to the Examiners and a discussion was had regarding the Nakamori reference and possible claim language that would differentiate the instant invention over the cited art, particularly the Nakomori reference.

Rejections Under 35 U.S.C. § 102

Claims 1-4 and 8-14 were rejected under 35 U.S.C. 102(b)as being anticipated by Nakamori et al.

This rejection is overcome, at least, for the following reasons.

Nakamori Cannot Anticipate Because It Does Not Teach A Directed Genetic Modification Or The Inhibition Of The Production Or Consumption Of A Substrate

The claims require generating a genetic modification in a gene of interest that inhibits the production or consumption of a metabolite by directed mutation. Nakamori, in contrast, uses random mutagenesis to generate hundreds of thousands (at least) of random mutagenic events. Nakamori then eventually identifies some cells in which an overexpressor phenotype of

methionine is identified. Further genetic analysis eventually finds that the repressor gene *metJ* is repressed. (Abstract, entire paper). As discussed by Nakamori, and the instant specification at for example, [0214], [0237], methionine and cysteine act in a negative feedback manner on the *metJ* gene to inhibit the production of methionine. When Nakamori mutates (randomly) the *metJ* gene, the result is the increased production of methionine by ***autotrophic*** superproducers on ***complete media*** increasing the production of methionine. See, pg. 181, col. 2, results and pg. 182, col. 1, especially, Table 2 and pages 183-185. There is no substrate or product that is inhibited. In Nakomori there is merely a de-repression of the repressor gene. This is succinctly stated in the instant specification at, for example, [0214]. This is also stated in Japanese patent application JP 2000157267 to Nakamori (believed to encompass the research of the Nakamori reference cited by the Office) and cited in the applicants Information Disclosure Statement. Further, this is stated in the Nakamori application (JP 2000157267) as the problem to be solved. "To obtain a new variation type *metJ* gene having a specific amino acid sequence lowered in activity in *repressing* methionine biosynthesis." JP 200015726 (Nakamori, Abstract). Therefore, Nakamori cannot anticipate the instant invention at least because the instant claims ***require*** "generating a genetic modification in a gene of interest wherein the production or consumption of a substrate is inhibited." As discussed in Nakamori, no production or consumption of any substrate is inhibited and only one of the, at least, hundreds of thousands (and more likely millions) of random mutagenic events generated 5500 were identified as analogue resistant and of these just seven were halo forming using the *pediococcus acidilactici* methionine assay. Of these seven only four produced appreciable quantities of methionine. Nakamori Table 1. Further, this assay provides a complete medium, excepting methionine, the production of which, by the inoculum, allows the *pediococcus acidilactici* to grow. See, Appendix I. Thus, the procedure of Nakamori is hardly directed genetic mutation and no cells (from the random mutagenic event) were selected in which the production or consumption of a substrate was inhibited. In fact, the opposite selection criteria were used, cells were selected in which the production was de-repressed. For these reasons alone, the rejection is overcome and should be withdrawn. Applicants respectfully request the same.

Nakamori Teaches Against the Instant Invention

As discussed above, the instant invention teaches a method of producing an evolved microorganism requiring, in part, the step of generating a microorganism wherein the production or consumption of a substrate is inhibited. In contrast, Nakamori explicitly states: "The construction of mutants that have a genetically altered regulatory mechanism ***is probably essential*** for the fermentative production of L-methionine." Pg. 179, 2nd column, last sentence (emphasis added). This statement is made with regard to the repressor genes *metJ* and *metK*. Thus, the ***essential*** requirement, as taught by Nakamori, is the removal of the negative feedback exerted by the *metJ* gene. There is no evolution of a "compensatory metabolic pathway" as is required by the instant claims. Therefore, for this reason alone, the rejection is overcome and should be withdrawn. Applicants respectfully request same.

The Process of Nakamori Does not Result in a Compensatory Metabolic Pathway

As discussed above and as required by the claims, the instant invention discloses the genetic modification of a gene of interest wherein the production or consumption of a metabolite is inhibited such that a compensatory metabolic pathway is evolved. In Nakamori, there is no difference in the pathway responsible for methionine synthesis other than the negative feedback mechanism is eliminated. No new substrates are available to the organism for methionine synthesis and no compensatory pathways are necessary (or indeed ever evolved). In Nakamori, the wild type pathway continues to produce methionine using pre-mutagenic synthesis steps. Thus, because the instant claims require the debilitation of a biosynthesis pathway and the evolution of a compensatory pathway, Nakamori cannot anticipate the instant invention. For this reason alone, the rejection is overcome and should be withdrawn.

Nakamori Teaches A Different Process Having Different Steps

As amended herein, the present invention claims a method for preparing an evolved microorganism comprising the steps of:

> a) generating a directed genetic modification in a gene of interest in an initial microorganism to yield a modified microorganism wherein the production or consumption of a substrate is inhibited when the modified microorganism is

grown on a defined medium, impairing the ability of the modified microorganism to grow;

b) culturing the modified microorganism obtained in step (a) on said defined medium, allowing the modified microorganism to evolve a compensatory metabolic pathway to compensate for the impaired growth, wherein the defined medium can contain a co-substrate promoting the evolution; and

c) selecting an evolved microorganism from step (b) able to grow on said defined medium;

wherein a compensatory metabolic pathway is evolved allowing the evolved microorganism to proliferate on the defined medium.

As described in the specification at, for example, [0011]-[0116], the genetic modification can at least include: (1) expressing a wild type gene that is otherwise suppressed; (2) expressing a heterologous gene that includes a sequence under the control of regulatory elements that permit its expression and translation in the original strain; (3) obtained by directed mutagenesis directly on the wild type gene present naturally in the initial strain, for example, by homologous recombination; or (4) obtained by other directed mutagenesis. Thus, those of skill in the art will appreciate that the ability to carry out any of the aforementioned procedures *requires* that the user identify and specifically determine which gene will be modified. For example, the process of homologous recombination cannot be performed without knowing the specific genetic sequence of the insertion point on the genome and the specific sequence of the genes being inserted. Further, such processes are taught in the specification at, for example, [0117]-[0120], as described in the specification. The "directed evolution" of the microorganism is then performed as described in the specification, at for example, [0231]-[0235] and in the Examples, provided from [0375] to [0421].

For example, these methods include a "coupling of the biosynthesis of the substance of interest with the growth of the micro-organism in such a way that the substance becomes necessary for the growth of the microorganism." [0232]. For example, for the production of methionine, the gene for methionine synthase is disrupted which results in the micro-organism becoming auxotrophic for methionine. As explained above, to survive in a minimum medium containing a simple carbon source and methyl mercaptan or sodium methyl mercaptide, the micro-organism has therefore to optimize the synthesis of L-methionine from O-acyl-L-homoserine and methyl mercaptan or sodium methyl mercaptide. [0234].

As explained in the specification, the induced auxotrophy results in an "evolved pathway" resulting from the "modified" activities of, for example, 'modified' methionine synthase [0069]-[0253], 'modified' cysteine synthase [0259]-[0307] and 'modified' NADPH synthesis [0308]-[0340] e.g. a compensatory pathway is evolved whereby at least one enzyme with a "modified activity results remediates the induced auxotrophy. See, FIGURES and EXAMPLES [0341]-[0653]. In these examples, the native activity of the initial non-evolved enzyme was not a methionine synthase, cysteine synthase, or NADPH synthase, it was, for example cystathionine γ-synthase that evolved into "methionine synthase", cystationine γ synthase that evolved into homocysteine synthase, O-acetyl-homoserine synthase that evolved into homocysteine synthase or O-acetyl-homoserine synthase that evolved into methionine synthase, for example. See, Fig. 2 and [0342]-[0350]. Thus, as explained it is possible to generate a genetic modification in a gene of interest e.g., "it is possible to obtain, ***in a controlled manner***, a modification of the substrate specificity of the enzyme" [0278] (emphasis added). As described, "[T]he strains modified according to the invention are genetically modified by the inactivation, mutation and/or over activation of at least one endogenous gene in order to permit the evolution of a new metabolic pathway" (e.g., a compensatory metabolic pathway). [0280]. As explained "the strain modified in this way is preferably selected and improved by a method of screening and evolution" making it possible, for example "to cause the acyl-homoserine sulfhydrylase activity to evolve into a cysteine synthase activity to restore the production of cysteine." [0284]. "The transformation of the acyl-homoserine sulfhydrylase activity into an "evolved cysteine synthase activity is deemed to be achieved when the genetically modified and evolved bacterial strain (E) has a growth rate at least similar to that of the initial modified strain (M) when grown in a minimal medium in the presence of glucose as a single carbon source." [0285]. Thus, in some exemplary embodiments, "the transformation of the acyl-homoserine sulfhydrylase activity into the "evolved cysteine synthase" activity is deemed to be achieved when the cysteine synthase activity carried by the modified O-acyl-L-homoserine sulfhydrylase protein has been improved by 10% relative to its initial activity. [0285]. Further methods of modification are provided in the specification at, for example, [0286]-[0292]. This is further succinctly stated in the Examples. "**b)** Culture of the above modified strain on the same minimal medium (MM) to which sodium methylmercaptide (co-substrate) has been added to cause the

evolution of an endogenous enzyme activity into a methionine-synthase activity to ***compensate*** for the initially deleted enzyme activity (*metE*)." [0365] (emphasis added).

It should be emphasized that after the genetic modification is generated, the modified microorganisms are grown in a non-minimal media to provide a volume of *the* mutant bacteria. Not, as in Nakamori, where innumerable random mutations are made and a desired mutant is then selected for. Thus, as explained at, for example, [0390]: "b) Culture and evolution of the Δ(*metE*) Modified Strain in the Presence of Sodium Methylmercaptide as Co-Substrate. To optimize E. coli for the production of methionine from methylmercaptan, a controlled selection is carried out in flasks. . . . The controlled selection is conducted in a hermetically sealed glass flask containing 50 ml of inorganic medium in the presence of 33mM glucose and chloramphenicol at a final concentration of 25mg/l. The culture media are seeded with the strain *E. coli* K12 Δ*metE* at a defined value of OD_{600}nm. Seeding is carried out with a sufficiently large population of bacteria so that some bacteria potentially possess relevant spontaneous mutations in the gene *metB* enabling assimilation of methylmercaptan (e.g., compensating for the auxotrophy as explained at, for example, [0250], [0302], [0363], [370], [0374], [0530]. This population is obtained by culture of the strain auxotrophic for methionine on a minimal medium supplemented with methionine." "[T]hree flasks then receive 100 µl of a 400 mg/l solution of sodium mercaptide, while a fourth flask received no added sodium mercaptide." [0396]. As discussed in the specification, the process of culturing and selecting the evolved microorganisms requires culturing the bacteria for a period of time long enough to allow the bacteria to develop the compensatory metabolic pathways, e.g., for 4-6 days or more. [0396]. As illustrated by TABLE 2, it was not until 6 days of growth that the modified microorganisms evolved enough to produce the desired end product. Further, applicants note that for wild type bacteria, a single overnight culture would yield an OD of over 1.0. Thus, the ability to produce the evolved bacteria requires the gentle culture of the bacteria over many "generations" of bacterial growth. As defined, Generate: to cause to exist; produce (Cambridge Dictionary of American English, © Cambridge University Press 2008.) Thus, there can be no doubt that producing a modified microorganism by directed genetic modification *a priori* requires generating a genetic modification in a gene of interest.

The instant method can then be summarized as follows:

1 – Directed genetic modification of a microorganism;

2 – Culturing only those micro organisms in which the directed genetic modification was made. Specifically, on a minimal media devised for the selection and evolutionary pressure of the desired biosynthetic pathway;

3 – Obtaining those microorganisms that grow in step 2.

Schematically, this procedure can be represented as:

$$\mathbf{I_o \rightarrow M_o \rightarrow E_o}$$

Where:

$\mathbf{I_o}$ is the initial microorganism;

$\mathbf{M_o}$ is the modified microorganism; and

$\mathbf{E_o}$ is the evolved microorganism.

In contrast, Nakamori does not teach "generating a directed genetic modification in a gene of interest" as required by the claims but rather, Nakamori teaches wild type E. coli that are mutagenized by N-methyl-N'nitro-N-nitrosoguanidine, *which induces random mutations*. Next, the mutagenized cells are spread onto plates containing various quantities of L-methionine analogs and incubated for 72 h. This step is performed to select cells that have acquired a mutation in the methionine biosynthesis pathway; however, this is not an evolutionary step. I.e., what Nakamori has done is merely randomly mutagenizing bacteria and selecting those that have become resistant to the L-methionine analogue ethionine. These resistant bacteria are then further selected for L-methionine overproduction.

The difference in Nakamori and the instant invention is succinctly illustrated in Fig. 1 of Nakamori as compared to Figs. 2 and 4 of the instant invention. As shown in Fig. 1 of Nakamori the native methionine biosynthetic pathway is intact merely the feedback inhibition has been eliminated. In contrast, as shown in Figs. 2 and 4 of the instant invention, induction of compensatory metabolic pathways (and enzyme) requires the disabling of the native gene (and enzyme). Thus, as summarized in Fig. 2, the native methionine biosynthetic pathway is the central pathway (corresponding to Nakamori, Fig. 1). Further, as shown in the figure and discussed in the specification, the step at which the compensatory pathway is desired

corresponds to the genetic mutation generated. Thus, when the *metC* gene is disabled the biosynthesis compensates with the evolution of either *metB*** or *metY**. When *metE* is disabled the biosynthesis compensates with an evolution of *metY*** or *metB**. Fig. 2, [0342]-[0350].

Thus, Nakamori can be summarized as follows:

1 – Random mutagenesis of bacteria

2 – Isolation of autotrophic superproducers on complete medium.

Schematically, this process can be represented as:

$$\mathbf{I_o \rightarrow R_o}.$$

Where:

$\mathbf{I_o}$ is the initial microorganism;

$\mathbf{R_o}$ is the randomly mutagenized microorganism.

Therefore, as discussed above, Nakamori cannot anticipate the instant invention, at least because the instant invention requires that the initial organism be modified by (1) a directed genetic modification such that (2) the production or consumption of a substrate is inhibited. Nakamori teaches neither of these elements. For at least these reasons, the rejection of claims 1-4 and 8-14 over Nakamori is overcome and should be withdrawn. Applicants respectfully request same.

Claims 1-7 Were Rejected Under 35 U.S.C. § 102(b) As Being Anticipated By WO 93/177112 To Lievense.

This rejection is overcome, at least for the following reasons.

As discussed above, the instant invention requires:

a) generating a directed genetic modification in a gene of interest in an initial microorganism to yield a modified microorganism wherein the production or consumption of a substrate is inhibited when the modified microorganism is grown on a defined medium, impairing the ability of the modified microorganism to grow;

b) culturing the modified microorganism obtained in step (a) on said defined medium, allowing the modified microorganism to evolve a compensatory

metabolic pathway to compensate for the impaired growth, wherein the defined medium can contain a co-substrate promoting the evolution; and

c) selecting an evolved microorganism from step (b) able to grow on said defined medium;

wherein a compensatory metabolic pathway is evolved allowing the evolved microorganism to proliferate on the defined medium.

In contrast, Lievense describes a method for enhancing methionine production in a fermentation process by transforming a microorganism with a homoserine-activating enzyme gene and a sulphur-incorporating enzyme gene. The aim of these genetic modifications is to improve the production of methionine by over-expressing **known genes,** supplied in trans, encoding for known enzymes involved in the biosynthesis pathways and deregulating the feedback mechanisms of those pathways. Pg. 6, second full paragraph. The process of Lievense does not teach a directed evolution process in which a "compensatory metabolic pathway" is induced in the microorganism but, rather, a process of identified genetic transformations. Further, applicants point out that Lievense does not teach the use of a defined culture medium but a culture medium containing glucose, soy hydrolysate, and inorganic nutrients. As discussed in depth in the previous response, soy hydrolysate **cannot** comprise a defined medium. Soy hydrolysate is a complex mixture of various soy components. The transformed cells of Lievense are not auxotrophic and would not be able to grow on a defined medium.

Specifically, the process of Lievense can be summarized as follows:

1 – Transforming cells with a homoserine-activating enzyme gene fragment and a sulfur-incorporating enzyme gene fragment; this is a step of "directed genetic modification corresponding to the over-expression of a heterologous gene described in the instant specification and reviewed above;

2 – Growing said cells in a medium for an undertermined period. This is not evolution but a rational genetic transformation;

3 – The resulting cells are collected and tested for L-methionine production.

The process of Lievense can be distinguished from the instant invention because *Lievense does not involve any step of evolution*. The transformed cells of Lievense are not subjected to

any "growing" period or allowed to "evolve" any compensatory pathway but, rather they are transformed with known genes to produce desired products using a well understood pathway that has been characterized (thus, the ability to transform with the desired genes) and grown in a complex medium simply for the purpose of producing L-methionine. In Lievense, there is no selective pressure and there is no directed genetic modification which results in the "inhibition of the production or consumption of a substrate." This is an element *required* of the claims. The Office is not free to read into Lievense the instant limitations. The microorganisms of Lievense are not grown in a defined medium and allowed to evolve new pathways to compensate for the autotrophic production of L-methionine. As autotrophs, there is no pressure to evolve any new pathways. These are very different conditions from the present invention.

The process of Lievense can be summarized as:

$$\mathbf{I_o} \rightarrow \mathbf{M_o}$$

Where

$\mathbf{I_o}$ is the initial microorganism;

$\mathbf{M_o}$ is the modified microorganism.

There are is no step of debilitating genes or biosynthetic pathways in the method of Lievense. Similar to the method of Nakamori, a repressor gene is de-repressed so that there is no negative feedback with respect to homoserine overproduction. (pg. 7, example 1). The cells are then merely transformed with plasmids encoding for the overproduction of the indicated compounds. In contrast to the present invention, where a compensatory pathway was developed using new substrates, in Lievense the same metabolic substrates as used by the wild-type enzymes are used for the overexpression. Lievense does not teach or even contemplate "the genetic modification of cells of an initial microorganism, so as to *inhibit* the production or consumption of a metabolite." This is a step that is **required** by instant claim 1. Lievense merely teaches the de-regulation of cells followed by the overproduction of the wild biosynthetic pathway wherein the same pathways and same substrates are used. Further, Lievense does not teach the attenuation of any gene involved in the production or consumption of a metabolite, *Lievense merely teaches the overexpression of a known heterologous gene.* This process is not at all analogous to the evolved expression of endogenous genes. Therefore, for these reasons alone,

the rejection over Lievense is overcome and should be withdrawn. Applicants respectfully request same.

In addition, while the rejection of claims 1-7 over Lievense have been overcome and should be withdrawn, Applicant has amended the claims to further include new claims 22 - 32. Support for claims 22-32 is explicitly found in the specification at, for example, paragraphs [0047]-[0340]. Various forms of directed genetic modification are usable with the present invention and many are described in the specification and reviewed above. No new matter is added by the amendments.

The Office Is Using Impermissible Hindsight Reconstruction To Identify The Required Elements Of The Claims

As stated above, the instant claims require:

a) generating a directed genetic modification in a gene of interest in an initial microorganism to yield a modified microorganism wherein the production or consumption of a substrate is inhibited when the modified microorganism is grown on a defined medium, impairing the ability of the modified microorganism to grow;

b) culturing the modified microorganism obtained in step (a) on said defined medium, allowing the modified microorganism to evolve a compensatory metabolic pathway to compensate for the impaired growth, wherein the defined medium can contain a co-substrate promoting the evolution; and

c) selecting an evolved microorganism from step (b) able to grow on said defined medium;

wherein a compensatory metabolic pathway is evolved allowing the evolved microorganism to proliferate on the defined medium.

A process rendered schematically as:

$$\mathbf{I_o \rightarrow M_o \rightarrow E_o.}$$

Lievense teaches a process rendered schematically as:

$$\mathbf{I_o \rightarrow M_o.}$$

The rejection of Claim 1 as being anticipated by Lievense is in error because the Office is improperly applying hindsight reconstruction to identify the claimed elements in the Lievense

application. Courts have repeatedly warned that the patentability of an invention is not to be viewed with hindsight or "viewed after the event." See Goodyear Co. v. Ray-O-Vac Co., 321 U.S. 275, 279, 64 S.Ct. 593, 88 L.Ed. 721 (1944) and authorities cited therein. The Office is further reminded of the warning recently provided by the Supreme Court and as further cited by the Board of Patent Appeals. "[A] factfinder should be aware, of course, of the distortion caused by hindsight bias and must be cautious of argument reliant upon ex post reasoning." KSR Int'l Co. v. Teleflex Inc., 127 S. Ct. 1727 at 1742.

Despite the lack of required elements, presence of media that are incompatible with the instant invention (i.e. soy hydrolysate and transformation by Lievense with known genes that thereby make the need for the required evolutionary steps of the instant invention irrelevant (and non functional)), the Office has identified elements in Lievense that simply are not present. The Office has not identified how "a fermentation medium containing glucose, soy hydrolysate, and inorganic nutrients" used by Lievense (Example 1) constitutes a "defined" medium, as is required by the claims. The Office has not identified how transformation "with plasmid(s) encoding homoserine acetyltransferase, O-acetylhomoserine (thiol)-lyase, and homocysteine methylase" (Example 1) constitutes the inhibition of the production or consumption of a substrate, as is required by the claims. The Office has not identified any compensatory metabolic pathway that would possibly be utilized by the bacteria transformed by Lievense, as is required by the claims. The construction of the present invention in the disclosure of Lievense is inherently *ex post facto*. "[A] factfinder should be aware, of course, of the distortion caused by hindsight bias and must be cautious of argument reliant upon *ex post* reasoning." KSR Int'l Co. v. Teleflex Inc., 127 S. Ct. 1727 at 1742. The rejection over Lievense while lacking required elements, is patently *ex post facto*. Thus, the rejection being overcome, withdrawal is respectfully requested.

The rejection over claims 1-7, in view of Lievense being now overcome, withdrawal of the rejection is respectfully requested.

CONCLUSION

This application now stands in allowable form and reconsideration and allowance is respectfully requested.

This response is being submitted on or before August 4, 2008 with the required fee of $1,050.00 for a 3-month extension of time, making this a timely response. It is believed that no additional fees are due in connection with this filing. However, the Commissioner is authorized to charge any additional fees, including extension fees or other relief which may be required, or credit any overpayment and notify us of same, to Deposit Account No. 04-1420.

Respectfully submitted,

DORSEY & WHITNEY LLP
Customer Number 25763

Date: July 25, 2008

By: 
Colin L. Fairman, Reg. No. 51,663
(612) 492-6864

Limitations of the Procedure

This prepared tube medium is intended to be used as an enrichment medium. A pure culture is recommended for biochemical tests and other identification procedures.

References

1. Gilligan, Janda, Kamnah and Miller. 1992. Gamiech 12A, Laboratory diagnosis of bacterial diarrhea. Coord. ed., Nolte. American Society for Microbiology, Washington, D.C.
2. Forbes, Sahm and Weissfeld. 1998. Bailey & Scott's diagnostic microbiology, 10th ed. Mosby, Inc., St. Louis, Mo.
3. Gramnich. 1992. In Isenberg (ed.), Clinical microbiology procedures handbook, vol. 1. American Society for Microbiology, Washington, D.C.

Availability

BBL™ Alkaline Peptone Water
Cat. No. 297814 Prepared Tubes, 8 mL (D Tubes) – Pkg. of 10

Amies Transport Media

(See Transport Media)

Amino Acid Assay Media
Lysine Assay Medium • Methionine Assay Medium
Cystine Assay Medium

Intended Use

Lysine Assay Medium is used for determining lysine concentration by the microbiological assay technique.

Methionine Assay Medium is used for determining methionine concentration by the microbiological assay technique.

Cystine Assay Medium is used for determining L-cystine concentration by the microbiological assay technique.

Summary and Explanation

Amino acid assay media are prepared for use in the microbiological assay of amino acids. Three types of media are used for this purpose:

1. Maintenance Media: For carrying the stock culture to preserve the viability and sensitivity of the test organism for its intended purpose;
2. Inoculum Media: To condition the test culture for immediate use;
3. Assay Media: To permit quantitation of the amino acid under test. They contain all the factors necessary for optimal growth of the test organism except the single essential amino acid to be determined.

Amino Acid Assay Media are prepared according to the formulations of Steel et al.[1] They are used in the microbiological assay of amino acids using *Pediococcus acidilactici* ATCC™ 8042 as the test organism.

Principles of the Procedure

Lysine Assay Medium, Methionine Assay Medium and Cystine Assay Medium contain all the factors essential for the growth of *Pediococcus acidilactici* ATCC 8042, except the amino acid under assay. The addition of the amino acid in specified increasing concentrations gives a growth response by the test organism.

Formulae[1]

Difco™ Lysine Assay Medium, Methionine Assay Medium or Cystine Assay Medium

All amino acid assay media contain the following formula.

Approximate Formula* Per Liter	
Dextrose	50.0 g
Sodium Acetate	40.0 g
Ammonium Chloride	6.0 g
Monopotassium Phosphate	1.2 g
Dipotassium Phosphate	1.2 g
Magnesium Sulfate	0.4 g
Ferrous Sulfate	20.0 mg
Manganese Sulfate	40.0 mg
Sodium Chloride	20.0 mg
Adenine Sulfate	20.0 mg
Guanine Hydrochloride	20.0 mg
Uracil	20.0 mg
Xanthine	20.0 mg
Thiamine Hydrochloride	1.0 mg
Pyridoxine Hydrochloride	2.0 mg
Pyridoxamine Hydrochloride	600.0 mg
Pyridoxal Hydrochloride	600.0 mg
Calcium Pantothenate	1.0 mg
Riboflavin	1.0 mg
Nicotinic Acid	2.0 mg
p-Aminobenzoic Acid	200.0 µg
Biotin	2.0 µg
Folic Acid	20.0 µg
Glycine	0.2 g
DL-Alanine	0.4 g
Asparagine	0.8 g
L-Aspartic Acid	0.2 g
L-Proline	0.2 g
DL-Serine	0.1 g
DL-Tryptophan	80.0 mg
L-Glutamic Acid	0.6 g
L-Histidine Hydrochloride	124.0 mg
DL-Phenylalanine	0.2 g
DL-Threonine	0.4 g
L-Tyrosine	0.2 g
DL-Valine	0.5 g
DL-Isoleucine	0.5 g
DL-Leucine	0.5 g
L-Arginine Hydrochloride	484.0 mg

User Quality Control

Identity Specifications

Difco™ Lysine Assay Medium, Methionine Assay Medium or Cystine Assay Medium

Dehydrated Appearance:	White to off-white, homogeneous, may have a tendency to clump.
Solution:	5.25% (single strength) solution, soluble in purified water upon boiling. Solution is light to medium amber, clear, may have a slight precipitate.
Prepared Appearance:	Single strength—Light to medium amber, clear, may have a slight precipitate
Reaction of 5.25% Solution at 25°C:	pH 6.7 ± 0.2

Cultural Response

Difco™ Lysine Assay Medium, Methionine Assay Medium or Cystine Assay Medium

Prepare the medium per label directions. These media support the growth of *Pediococcus acidilactici* ATCC™ 8042 when prepared in single strength and supplemented with the appropriate amino acid. Lysine Assay Medium should produce a standard curve when tested with L-Lysine at 0.0 to 300 µg per 10 mL. Methionine Assay Medium should produce a standard curve when tested with DL-Methionine at 0.0 to 60 µg per 10 mL. Cystine Assay Medium should produce a standard curve when tested with L-Cystine at 0 to 50 µg per 10 mL. Incubate tubes with caps loosened at 35-37°C for 16-20 hours. Read the percent transmittance at 660 nm.

Preparation of inoculum dilution, amino acid stock and working solution.

ASSAY MEDIUM	TEST CULTURE	PREPARATION OF INOCULUM DILUTION (CELL SUSPENSION + STERILE 0.85% NACl)	PREPARATION OF AMINO ACID STOCK SOLUTION (AMINO ACID + PURIFIED H_2O)	STANDARD WORKING SOLUTION (5 TOCC SOLUTION + PURIFIED H_2O)	VOLUME OF STANDARD WORKING SOLUTION (ML/10 ML TUBE)	FINAL AMINO ACID CONCENTRATION µG/10 ML
Cystine Assay Medium	*Pediococcus acidilactici* ATCC™ 8042	1 mL + 19 mL	L-cystine 1 g + 100 mL + 1 mL HCl heated, then cooled, add up to 1,000 mL	1 mL + 99 mL	0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5	0.0, 5, 10, 15, 20, 25, 30, 40, 50
Lysine Assay Medium	*Pediococcus acidilactici* ATCC™ 8042	1 mL + 19 mL	L-lysine 6 g + 1,000 mL	1 mL + 99 mL	0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5	0.0, 30, 60, 90, 120, 150, 180, 240, 300
Methionine Assay Medium	*Pediococcus acidilactici* ATCC™ 8042	1 mL + 19 mL	DL-methionine 1.2 g + 1,000 mL	1 mL + 99 mL	0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5	0.0, 6, 12, 18, 24, 30, 36, 48, 60

In addition to the ingredients listed on the previous page, the media contain per liter*:

Lysine Assay Medium		
L-Cystine	0.1	g
DL-Methionine	0.2	g
Methionine Assay Medium		
L-Cystine	0.1	g
L-Lysine Hydrochloride	0.5	g
Cystine Assay Medium		
DL-Methionine	0.2	g
L-Lysine Hydrochloride	0.5	g

*Adjusted and/or supplemented as required to meet performance criteria.

Precautions

Great care must be taken to avoid contamination of media or glassware in microbiological assay procedures. Extremely small amounts of foreign material may be sufficient to give erroneous results. Scrupulously clean glassware free from detergents and other chemicals must be used. Glassware must be heated to 250°C for at least 1 hour to burn off any organic residues that might be present. Take precautions to keep sterilization and cooling conditions uniform throughout the assay.

Directions for Preparation from Dehydrated Product

1. Suspend 10.5 g of the powder in 100 mL of purified water.
2. Heat with frequent agitation and boil for 2-3 minutes to completely dissolve the powder.
3. Dispense in 5 mL amounts into tubes, evenly dispersing the precipitate.
4. Add standard or test samples.
5. Adjust tube volume to 10 mL with purified water.
6. Autoclave at 121°C for 10 minutes.

Procedure

Stock Culture and Inoculum

Stock cultures of *Pediococcus acidilactici* ATCC 8042 are prepared by stab inoculation into tubes of Lactobacilli Agar AOAC or Micro Assay Culture Agar. Incubate cultures at 35-37°C for 24 hours. Store stock cultures at 2-8°C. Make transfers at monthly intervals in triplicate.

The inoculum for assay is prepared by subculturing the test organism into 10 mL Lactobacilli Broth AOAC or Micro Inoculum Broth. Incubate at 35-37°C for 16-24 hours. After incubation, centrifuge the cells under aseptic conditions and decant the liquid supernatant. Wash the cells 3 times with 10 mL sterile 0.85% NaCl solution. After the third wash, resuspend the cells in 10 mL sterile 0.85% NaCl solution. Dilute the 10 mL cell suspension with the appropriate amount of sterile 0.85% NaCl solution. (See the table under User Quality Control, Cultural Response.) One drop of the diluted inoculum suspension is used to inoculate each of the assay tubes.

Amino Acid Solution

Prepare stock solutions of each amino acid as described in Table 1. If the DL form is used, twice the concentration of the amino acid is required. Prepare the stock solutions fresh daily.